DIAGNOSTIC AND THERAPEUTIC MEANS FOR PATHOLOGIES ASSOCIATED WITH ALPHA 2 SUBUNIT OF THE NA,K PUMP

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5   The present invention concerns diagnostic and therapeutic means for pathologies associated with alpha 2 subunit of the Na,K pump, as migraine and alternating hemiplegia of the childhood.

Migraine is characterized by headache attacks and is sometimes associated with autonomic nervous system dysfunctions and transient neurological symptoms (aura). The prevalence of migraine in the general population is

10   12%, and 20% of patients present with aura (1—5).

Although the mode of transmission is controversial (6), migraine shows strong familial aggregation (7). Several population-based and twin-based studies have indicated that genetic factors are implicated, particularly in migraine with aura (8, 9). Familial hemiplegic migraine (FHM) is a disabling

15   neurological disease that manifests with aura and hemiparesis. It affects about 1/10,000 to 1/50,000 individuals and is transmitted as an autosomal dominant trait (10).

A gene associated with FHM1(MIM 141500) and encoding a neuronal calcium channel protein (CACNA1A) has previously been identified (11).

20   The PCT patent application WO98/55647 describes an indirect genotyping method for diagnosing hemiplegic migraine type 2 that concerns a wide region of 21 cM (centimorgan) of chromosome 1q21-23. The description does not identify any genes associated with the disease but suggests two candidate genes, GIRK3 (encoding a potassium channel protein) and

25   CACNL1A6 (encoding a calcium channel protein).

The authors of the present invention have identified the gene associated with FHM2 (MIM 602481) that maps on chromosome 1q23 (12) and have shown that mutations in the alpha 2 subunit of the Na,K ATPase pump (ATP1A2) are responsible for the disease. The identified gene does not correspond to

30   any genes or regions suggested in the previous technical documentation, particularly as regards the aforesaid patent application WO98/55647. The

authors have demonstrated that the identified missense mutations cause a loss-of-function of the major ion transport system. This has relevant implications for the origin of cortical spreading depression of neuronal disease and the development of migraine. It is the first demonstration that

5   mutations in the Na,K pump are associated with genetic diseases. It should also be stressed that a study on GIRK3 and CACNL1A6 have not shown any mutations in these genes that could be correlated with migraine. Furthermore, it is possible to study polymorphisms associated with said gene to correlate them as predisposing factors for common migraine.

10   Alternating hemiplegia of the childhood (AHC, OMIM 104290) is a rare syndrome (estimated prevalence 1 in 1.000.000), characterized by early onset of episodic hemi- or quadriplegia lasting minutes to days. Mutation analysis in the ATP1A2 gene was performed by direct sequencing of all exons with the same primers used for amplification. An heterozygous

15   mutation (1237 C->A) segregating with the disease in a AHC family and causing a threonine to asparagine replacement (T378N) was found. This mutation is not present in any of the unaffected members of the family and in 250 control chromosomes. Hence, the object of the present invention is a nucleic acid comprising at

20   least one segment of the gene encoding a functional portion or gene-regulating region of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in the diagnosis of pathologies associated with migraine or with the alternating hemiplegia of the childhood. A further object of the invention is a nucleic acid comprising at lease one

25   segment of the gene encoding a functional portion or gene-regulating region of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in genetic therapy for pathologies associated with migraine or with the alternating hemiplegia of the childhood. A further object of the invention is a method to detect in an individual at least

30   one mutation of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) located on chromosome1, associated with

migraine disorders or with the alternating hemiplegia of the childhood, which comprises the steps of: - collecting a sample containing a sufficient quantity of the individual's DNA or that is reproducible in culture; - isolating the DNA from the sample; - exponentially amplifying the DNA using as an

5   oligonucleotide pair for the amplification reaction at least two oligonucleotides that are able to amplify at least one segment of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) or of a segment of the region regulating it; - detecting in at least one amplified segment any mutations compared with a healthy control. Preferably, the oligonucleotide

10   pairs are:

     17   AGTCCCTCTGACCTCCCTGAT   CCACTGTGCCATCACGATT
     19   CTTCTGCTTCCTGCTCTGACC   ACACATGTGCGCTGTGTTTAC.

In an embodiment of the method of invention, the DNA exponential amplification phase is performed using oligonucleotide pairs that are able to

15   amplify the entire encoding portion of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2). Preferably, the DNA exponential amplification phase to amplify the entire encoding portion of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) comprises the use of at least one of the following oligonucleotide

20   pairs:

     1 TGTTGCTTTGGCTTTCTCTGT   CTCCCTCACCCTCTAGACTGC
     2+3 CCCCTCTCTTCCCTGACTCT   GCCTCTTTTGTTCCTTCCCTA
     4 ATGGTGACTGGCTGGGTTG   CAGGGTTGGAGGACAGTCAC
     5 AGCTGCCCCTTTAGGGTTG   ACCTTACAGCCTAGCCCAGAG

25   6 GAGACCAGCAGGAGAAGAAGG   AGACTCAACTGCTTGCTCTGG
     7 TACAAGTGGCTCTGCCAGTCT   AGCCCTTCATCCTGACTATGG
     8 CAGGAAATAGGATGGGACTGC   GTAGTGAGACCCTCCCCTGGT
     9 ATCTCCGGCTTCAGCCTTAAC   TAATCCTATCCACCCCCTCTG
     10+11 CTCCTGGTTCCCCCTCAT   TCCCTCTCTCTTCCTCTGTCC

30   12 GCGCTACCAAGACAAGTATGG   CTTGGGAATCCCCTTCTGAG
     13 GAAGCCACTCTGCGGATCT   ACTGCAGCTCCTTGAACTCTG
     14 GGAGGGGGATAAACCCTTAAT   GACGTGTTGATTAGGGCACAG
     15 AGGGGTCAGCTGTCTCTGTC   GGTCCCTGCCTGTCATCTG

16 AAGGGGTTTCGTCCTCAAGT   TCAGTATCCTGCAAACCATCC
17 AGTCCCTCTGACCTCCCTGAT   CCACTGTGCCATCACGATT
18 TCATCTCCTACGTCCCTTCAA   AGCTGGGAAAAGAACCCTGT
19 CTTCTGCTTCCTGCTCTGACC   ACACATGTGCGCTGTGTTTAC
20 CCTCCGACACTCTCATCTGTC   CTGTGTGGGTTGGTGAGTGT
21 CTTCACCTGCCACCTCCTT   CCCCCGTATGACTACTCAGG
22 CGCTTTGAATGCTCCTTTATG   GAGGGAGGAGCTGGTGGT
23 GCCTCCTTTTAAGCTCATGCT   GCCTCATTATCTCTCCCCAAA

In an embodiment of the method of invention, the DNA exponential
amplification phase is performed using oligonucleotide pairs that are able to
amplify the regulating region of the gene encoding the alpha 2 subunit of the
Na,K human pump (ATPase, ATP1A2). Preferably, the DNA exponential
amplification phase to amplify the regulating region of the gene encoding the
alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) comprises the
use of the following oligonucleotide pairs:

1_Pr   TTCCCCTCACTCCATCTCTG   GACCCCTGCTCTTTAGGGATA
2_Pr   GATTCAGGACCACTCCATCC   GGGAACAGTCAGAGGACAGG

In a preferred embodiment of the method of the invention, the detection
phase of at least one amplified segment with any mutations compared with a
healthy control is performed using direct sequencing or an SSCP method
(single strand conformation polymorphism) (17) DHPLC or DGGE
(denaturing gradient gel electrophoresis) (18) or other methods known to an
expert from the field.

A further object of the invention is a diagnostic kit for pathologies associated
with migraine or with alternating hemiplegia of the childhood which
comprises: - at least a pair of oligonucleotides for the exponential
amplification reaction of at least one segment of the gene encoding the alpha
2 subunit of the Na,K human pump (ATPase, ATP1A2), in which the segment
encodes a functional portion or a gene-regulating portion of the subunit; - a
control DNA from a non affected individual. In a preferred form, the
oligonucleotide pairs for the amplification reaction are able to amplify the

entire encoding region of the gene encoding the alpha 2 subunit of the Na,K
human pump (ATPase, ATP1A2).

A further object of the invention is the alpha 2 subunit protein of the Na,K
human pump (ATPase, ATP1A2) or a functional portion thereof for use in the

5   diagnosis of pathologies associated with migraine or with alternating
hemiplegia of the childhood.

A further object of the invention is the alpha 2 subunit protein of the Na,K
human pump (ATPase, ATP1A2) or a functional portion thereof for use in the
treatment of pathologies associated with migraine or with alternating

10   hemiplegia of the childhood.

A further object of the invention is a method for the identification of an agonist
or antagonist agent of the Na,K human pump (ATPase, ATP1A2) or a
functional portion or a gene-regulating portion of thereof, that comprises:

(i) transfection of a cell line with a gene for a mutant isoform of the Na,K

15   human pump (ATPase, ATP1A2) resistant to oubain;

(ii) appropriate exposure of the transfected cells to the agent;

(iii) measurement of the Na,K pump activity in relation to ion transport with
labelled ions.

A further object of the invention is a method to identify an agonist or

20   antagonist agent of the Na, K pump (ATPase, ATP1A2) or a functional
portion, that comprises the phases:

(i) use of an agent to treat a transgenic animal that expresses a mutant
isoform of the Na,K pump (ATPase, ATP1A2) or that partially or completely
deletes the gene encoding the Na,K pump (ATPase, ATP1A2) or

25   (ii) use of an agent to treat eukaryotic or prokaryotic cell lines that express
mutant or normal forms of the Na,K pump (ATPase, ATP1A2) by transient or
stable transfection or in physiological conditions.

The invention is described below in reference to non limiting examples and
the following figures:

30   Figure 1. ATP1A2 mutation detection. Panel a, the normal (blue) and mutant
(red) D-HPLC elution patterns of exon 17 (left) and 19 (right); Panel b shows

the direct- sequencing electropherograms of the control (upper part) and mutant heterozygotes (lower part); Panel c, the pedigree of the two FHM2 families.

Figure 2. Local amino acid sequence alignment of ATPases. The complete conservation of L764 (left) and W887 (right) in several subunits of Na,K ATPases and H,K ATPases is shown. The relative SwissProt accession number is indicated.

Figure 3. ATP1A2 protein topology. The ouabain binding site on the first loop (M1-M2; asterisks indicate the mutagenized amino acids to confer ouabain resistance) and the two mutations on the largest intracellular (M4-M5) and extracellular (M7-M8) loops are highlighted.

Figure 4. Ouabain treatment of transfected HeLa cells. Phase contrast pictures of HeLa cells taken after 36 hr of 1 μM ouabain challenge transfected with: panel a, mock transfected cells (a construct expressing wild-type ATP1A2 non-ouabain resistant was used); panel b, a ouabain resistant wild-type ATP1A2, pA2Oua^r-wt; panels d and f, ouabain resistant ATP1A2 mutants, pA2Oua^r-P764 and pA2Oua^r-R887, respectively; panel c, a 1:1 mixture of pA2Oua^r-wt + pA2Oua^r-P764, to simulate the L764P heterozygous state; panel e, a 1:1 mixture of pA2Oua^r-wt + pA2Oua^r-R887, to simulate the W887R heterozygous state. All experiments were performed by co-transfecting an ATP1B2 expressing construct.

Figure 5. Time course of ouabain toxicity. Panel a, cell viability by MTT assay of HeLa cells transfected with different constructs as reported in Figure 4: mock; A2-wt (pA2Oua^r-wt); mu-1 (pA2Oua^r-P764); het-1 (pA2Oua^r-wt + pA2Oua^r-P764); mu-2 (pA2Oua^r-R887); het-2 (pA2Oua^r-wt + pA2Oua^r-R887). Both mutants and simulated heterozygotes are significantly different from A2-wt (at least P< 0.04). Bars represent SD. Panel b, in vitro transcription and translation confirming the expected molecular mass of ATP1A2 protein of 112 kDa.

Figure 6. Localization of mutant ATP1A2 to the plasma membrane. Panel a, immunocytochemistry on COS-7 cells of the c-myc-derivatives, pA2Oua^r-wt-

myc, pA2Oua^r^-P76*4-myc* and pA2Oua^r^-R88*7-myc*, showing the plasma membrane localization of both wild type and mutant isoforms. Panel b, subcellular fractionation of transfected COS-7 cells demonstrating the plasma membrane co-sedimentation with ATP1A2 *c-myc*-derivatives; s/n,

5   supernatant; p, pellet.

Figure 7. Phase-contrast pictures of transfected HeLa cells taken after 72h of treatment with 1 μM ouabain. a, transfection with a cDNA construct expressing non-ouabain-resistant wild-type ATP1A2. b, transfection with a cDNA construct expressing ouabain-resistant wild-type ATP1A2. c,

10   transfection with a cDNA construct expressing ouabain-resistant T328N ATP1A2 mutant. d, transfection with a 1:1 mix of constructs expressing ouabain-resistant wild-type ATP1A2 and T328N ATP1A2 mutant to simulate the hetrozygous state.

Figure 8. Cell viability assessed by MTT assay of the transfected HeLa cells

15   shown in Fig. 7. Y axis represents the percentage of surviving cells

EXAMPLE 1 Migraine

Materials and Methods

FHM2 families

Twenty-two subjects from a large Italian pedigree (family 1), originating from

20   Tuscany, with a clinical diagnosis of FHM (5) and seven members with similar manifestations from an unrelated pedigree (family 2) from Sicily, were selected. No cerebellar signs were associated with FHM. The onset of attacks always occurred by the age of twenty. Additional features were history of seizures in five members (three subjects from family 1 and two

25   subjects from family 2) and mild or moderate mental retardation in two subjects from family 1. Two hundred randomly collected healthy individuals from the Italian population were used as control subjects.

Mutation screening

We determined the genomic organization of the human *ATP1A2* gene by

30   aligning the sequence of *ATP1A2* mRNA (AC NM_000702) with the corresponding genomic sequence on clone RP11-536C5. Designed

oligonucleotide primers for amplification of the gene encoding regions are reported in Table 1. PCR products that showed an abnormal D-HPLC (Wave, Transgenomic, Crewe, UK) retention patterns were subjected to direct sequencing (DYEnamic ET Dye Terminator Kit, Amersham Biosciences, Piscataway, NJ, USA).

Table 1

Exon	forward primer	reverse primer	bp
1	TGTTGCTTTGGCTTTCTCTGT	CTCCCTCACCCTCTAGACTGC	177
2+3	CCCCTCTCTTCCCTGACTCT	GCCTCTTTTGTTCCTTCCCTA	423
4	ATGGTGACTGGCTGGGTTG	CAGGGTTGGAGGACAGTCAC	316
5	AGCTGCCCCTTTAGGGTTG	ACCTTACAGCCTAGCCCAGAG	213
6	GAGACCAGCAGGAGAAGAAGG	AGACTCAACTGCTTGCTCTGG	238
7	TACAAGTGGCTCTGCCAGTCT	AGCCCTTCATCCTGACTATGG	234
8	CAGGAAATAGGATGGGACTGC	GTAGTGAGACCCTCCCCTGGT	385
9	ATCTCCGGCTTCAGCCTTAAC	TAATCCTATCCACCCCCTCTG	283
10+11	CTCCTGGTTCCCCCTCAT	TCCCTCTCTCTTCCTCTGTCC	487
12	GCGCTACCAAGACAAGTATGG	CTTGGGAATCCCCTTCTGAG	284
13	GAAGCCACTCTGCGGATCT	ACTGCAGCTCCTTGAACTCTG	286
14	GGAGGGGGATAAACCCTTAAT	GACGTGTTGATTAGGGCACAG	236
15	AGGGGTCAGCTGTCTCTGTC	GGTCCCTGCCTGTCATCTG	284
16	AAGGGGTTTCGTCCTCAAGT	TCAGTATCCTGCAAACCATCC	284
17*	AGTCCCTCTGACCTCCCTGAT	CCACTGTGCCATCACGATT	252
18	TCATCTCCTACGTCCCTTCAA	AGCTGGGAAAAGAACCCTGT	234
19*	CTTCTGCTTCCTGCTCTGACC	ACACATGTGCGCTGTGTTTAC	232
20	CCTCCGACACTCTCATCTGTC	CTGTGTGGGTTGGTGAGTGT	236
21	CTTCACCTGCCACCTCCTT	CCCCCGTATGACTACTCAGG	176
22	CGCTTTGAATGCTCCTTTATG	GAGGGAGGAGCTGGTGGT	223
23	GCCTCCTTTTAAGCTCATGCT	GCCTCATTATCTCTCCCCAAA	206

The primer pairs 17 and 19 (*) were used to identify the two mutations associated with FHM2. The PCR were designed with a uniform annealing temperature of 57° C.

The oligonucleotides that permit the amplification of the gene expression regulating regions (about 3 kb, subdivided in two partially overlapping segment) are listed in Table 2.

5   Table 2

1_Pr	TTCCCCTCACTCCATCTCTG	GACCCCTGCTCTTTAGGGATA
2_Pr	GATTCAGGACCACTCCATCC	GGGAACAGTCAGAGGACAGG

The analysis of the amplified DNA was performed using direct sequencing
10   and DHPLC (denaturing high-pressure chromatography (16).

Constructs and site-directed mutagenesis

The full-length cDNA coding for the beta 2 (ATP1B2, NM_001678) and alpha 2 were derived from IMAGE clone 23453 and DFKZp761D047, respectively, and subcloned in the expression vector pcDNA3.1 (Invitrogene, Carlsbad,
15   CA, USA). We used the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) for mutagenizing the *ATP1A2* cDNA as follows:

a) nt451 A>G (Q116R) and nt483 A>G (N127D) to obtain the construct pA2Ouaʳ-wt expressing the ouabain-resistant isoform;
20   b) nt2395 T>C (L764P) on pA2Ouaʳ-wt, obtaining pA2Ouaʳ-P764;

c) nt2763 T>C (W887R) on pA2Ouaʳ-wt, obtaining pA2Ouaʳ-R887.

d) *c-myc*-tagging the *ATP1A2* cDNA. Expression constructs (pA2Ouaʳ-wt, pA2Ouaʳ-P764, and pA2Ouaʳ-R887) were mutagenized by replacing the original start codon with the *c-myc* tag (consisting of aa MAEEQKLISEEDL,
25   corresponding to aa 408-419 of the human c-myc AC 0907235A) obtaining pA2Ouaʳ-wt-*myc*, pA2Ouaʳ-P764-*myc* and pA2Ouaʳ-R887-*myc*. All constructs were sequence-verified.

In vitro transcription and translation

In vitro transcription and translation was performed using the TNT Coupled
30   Reticulocyte Lysate System (Promega, Madison, WI, USA) in the presence of 20 microCi [^{35}S] methionine (1000 Ci/mmole) and neosynthesized proteins were separated by SDS/PAGE (8%).

Electrophoresis and western blot analysis

Equal amounts of proteins were resuspended in SDS-PAGE buffer (62.5 mM Tris- HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and separated for 2 h at 100V in 10% SDS-polyacrylamide gels. Transblotted
5   nitrocellulose membranes were incubated with monoclonal primary antibodies anti *c-myc* 9E10 (10 µg/ml) or polyclonal antibodies anti-Integrin beta 1 followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized with the Enhanced Chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

10 Transfections and ouabain treatment

Constructs were transfected by calcium phosphate (HeLa cells) following standard procedures or by Lipofectamine (COS-7 cells), (Gibco, Invitrogene Co., Carlsbad, CA, USA).

Cell viability was measured with the MTT (13) reduction assay after 12, 24,
15 36, 48 and 60 hours. Each experiment was made in triplicate and statistical analysis was done by Student's *t*-test (homoscedastic).

Immunocytochemistry

After transfection (48 hr), COS-7cells were fixed in 100% methanol and incubated with monoclonal primary antibodies anti c-*myc* 9E10. Cells were
20 then washed with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA). Cells were coverslipped in fluorescent mounting medium (DAKO, Glostrup, Danmark) and visualized under epifluorescence optics.

Subcellular fractionation

25 COS-7 cells were lysated on ice in 0.5 M NaCl, 10 mM NA2C03, 0.1 mM PMSF, 10 µg/ml Aprotenin, 10 µg/ml Leupeptin, homogenated and centrifuged at 2000 g for 20 min at 4°C to discard nuclei and cellular debris. Separation of the membrane fraction (pellet) from the cytosolic fraction (supernatant) was achieved by centrifugation at 100,000 g for 40 min at 4°C
30 in a Beckman TL 100 ultracentrifuge.

Results

Mutation analysis

Although the reduced FHM2 critical region spanned only 0.9 Mb between D1S2635 and CASQ1-SNP, several positional candidate genes, which are expressed in the central nervous system, are present in this genomic area.

5   We performed mutation analysis on two probands of the FHM2 families by both D-HPLC (denaturing HPLC) and direct sequencing on the two potassium channel genes, *KCNJ9* and *KCNJ10* and the *CASQ1* gene coding for calsequestrin with negative results.

In contrast, D-HPLC mutation scanning of the *ATP1A2* gene encoding the

10   alpha 2 subunit of the Na,K ATPase gave aberrant elution patterns for exon 17 and exon 19 in families 1 and 2, respectively (Fig. 1a). Sequencing analysis revealed the presence of two point mutations (nt 2395 T to C and nt 2763 T to C; Fig. 1b), each segregating with the disease in the respective families (Fig.1c) and causing the amino acid replacements leucine to proline

15   (L764P) in family 1 and tryptophan to arginine (W887R) in family 2. Both missense mutations were absent in 400 control chromosomes. L764 and W887 are completely conserved among alpha subunits from several evolutionary distant species (Fig. 2), thus strongly suggesting a causal role of ATP1A2 mutations in the pathogenesis of FHM2.

20   The Na,K pump is a heterodimeric structure formed by a large catalytic alpha subunit and a small ancillary beta subunit. The alpha subunits traverse the plasma membrane with 10 transmembrane segments (M1-M10) (14) and expose the amino- and carboxy-termini towards the cytoplasm. This configuration assigns five extracellular and four intracellular loop domains.

25   L764P and W887R mutations have different localization within the alpha topology: L764P maps to the large intracellular loop between M4 and M5, while W887R localizes to the apical M7-M8 loop (Fig. 3).

Functional evidence of impaired ion transport

In order to evaluate the functional consequences of these two amino acid

30   replacements involving different protein structures, we carried out various transfection experiments. We cloned the full-length human cDNAs of the

alpha 2 and beta 2 subunits (see Materials and Methods section). Since both subunits are required for assembling the active alpha-beta heterocomplex, all transfection experiments hereafter were carried out by co-transfecting alpha 2 and beta 2 constructs with equal stoichiometry. By introducing the mutant

5   isoforms (i.e. expressing the mutant P764 and R887 full-length cDNAs), we obtained no significant changes in the cell shape or growth rate (data not shown), thus excluding a primary dominant-negative effect.

As all vertebrate cells present Na,K ATPase activity, the endogenous Na,K pump activity of HeLa cells was quenched to test the ion transport

10   performance of the exogenous mutant forms. A site-directed mutagenesis was carried out to abolish the natural ouabain sensitivity of the ATP1A2 construct by mutagenizing two amino acids, Q116R and N127D, in the first extracellular loop that is part of the ouabain binding site of Na,K ATPase (15). HeLa cells transfected with the ouabain-resistant ATP1A2 cDNA construct

15   (pA2Oua^r-wt) can survive and grow in 1 μM ouabain-containing media (Fig. 4, panel b), while mock transfected cells die within 36--48 hours (Fig. 4, panel a). Identical results were obtained with the human cell line HEK293.

Once positively tested for ouabain resistance, the pA2Oua^r-wt construct was subsequently mutagenized to introduce the FHM2 mutations L764P and

20   W887R, obtaining the corresponding constructs pA2Oua^r-P764 and pA2Oua^r-R887. HeLa cells transfected with pA2Oua^r-P764 or pA2Oua^r-R887 failed to survive 1 μM ouabain treatment (Fig. 4, panels d and f), thus suggesting that both L764P and W887R are loss-of-function mutations.

Simulated heterozygous states obtained by co-transfecting equal amounts of

25   wild-type and mutant constructs showed an intermediate behavior (Fig. 4, panel c and e). Both mutant ATP1A2 isoforms showed early cell mortality typical of cells lacking Na,K pump activity (Fig. 5a). To exclude the possible production of aberrant proteins from site-directed mutagenesis, we tested the wt and mutant constructs by in vitro transcription and translation experiments

30   and direct sequencing. As shown in Figure 5b, all three constructs gave the expected 112 kDa protein band, thus excluding the possibility that a cloning

artifact is responsible for the vulnerability of HeLa cells to ouabain when transfected with the mutant *ATP1A2* cDNAs.

Mutant ATP1A2 isoforms are delivered to the plasma membrane

We investigated the subcellular localization of the two mutant isoforms. The

5   three constructs (pA2Oua^r-wt, pA2Oua^r-P764, and pA2Oua^r-R887) were engineered by adding a 5' tag coding for the *c-myc* epitope and transfected into COS-7 cells. Figure 6a shows the expected immunofluorescence localization to the plasma membrane of all isoforms, both wild type and the two mutants. Subcellular fractionation confirmed the physiological location in

10   the membrane fraction (Fig. 6b), where the integrin beta 1 subunit was detected as a control.

These data demonstrate that both missense mutations are independently sufficient to inhibit Na,K pump activity, without affecting the assembling with the beta subunit and the complex translocation to the cell membrane.

15   **Example 2 <u>Alternating hemiplegia of the childhood</u>.**

Materials and Methods

Constructs and site-directed mutagenesis.

The full length cDNA coding for the alpha 2 was subcloned in the expression vector pcDNA3.1 (Invitrogene, Carlsbad, CA, USA). We used the

20   QuickChange site-directed mutagenesis kit (Stratagene, La lolla, CA, USA) for mutagenizing the *ATP1A2* cDNA as follows:

Q116R and N127D to obtain the construct pA2Oua^r-wt expressing the ouabain resistant isoform;

T378N on pA2Oua^r-wt, obtaining pA2Oua^r-AHC mutated form;

25   All constructs were sequence-verified.

Transfections and ouabain treatment.

Constructs were transfected by calcium phosphate (HeLa cells) following standard procedures.

Cell viability was measured with the MTT reduction assay after 24, 48 and 72

30   hours of 1 µM ouabain challenge. We performed two independent experiments and in each experiment, datapoints are in triplicate.

Results

Alternating hemiplegia of the childhood (AHC, OMIM 104290) is a rare
syndrome (estimated prevalence 1 in 1.000.000), characterized by early
onset of episodic hemi- or quadriplegia lasting minutes to days.

5   Mutation analysis in the ATP1A2 gene was performed by direct sequencing
of all exons with the same primers used for amplification. An heterozygous
mutation (1237 C->A) segregating with the disease in a AHC family and
causing a threonine to asparagine replacement (T378N) was found. This
mutation is not present in any of the unaffected members of the family and in

10   250 control chromosomes.

This missense mutation localizes to the ATPases phosphorylation site
(DKTGTLT, aa 374-380) of the hydrolase domain of the protein. In the □□
subunit topology the mutated residue resides in the large intracellular loop
within the M4-M5 transmembrane segments (M1-M10, [Hu, 2000 #7823]).

15   The affected residue is highly conserved in all the known □ subunits of the
Na,K pump from vertebrates to invertebrates suggesting a functional role in
pump activation.

To evaluate the functional consequences of this mutation we carried out
transfection experiments in human HeLa cells. Since all mammalian cells

20   have Na+/K+ ATPase, we quenced the endogenous pump activity using
ouabain, and tested the function of the exogenously transfected mutant of
cDNA constructs engineered to be resistant to ouabain. Using site-directed
mutagenesis we introduced two amino acid changes (Q116R and N127D) in
the first extracellular loop to confer resistance to ouabain, and the AHC

25   mutation T378N. HeLa cells transfected with this construct did not survive to 1
μM ouabain treatment. A simulated heterozygous state, as obtained by
transfecting equal amount of wild-type and mutant cDNAs, showed
intermediate behaviour (Fig. 7).

In addition, as revealed by time course experiments, the mutant isoform

30   show rapid mortality typical of cells lacking Na^+/K^+ ATPase pump activity
(Fig. 8).

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